THE FAILURE OF HYPOPHYSECTOMIZED FUNDULUS HETEROCLITUS TO SURVIVE IN FRESH WATER

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A pituitary control of osmoregulation in *Fundulus heteroclitus* was first suggested by Pickford (1953). She noted that six hypophysectomized fish which had been placed in fresh water were unable to survive, although such operated fish would live indefinitely in sea water and normal *Fundulus* will withstand abrupt changes of salinity with no adverse effects. These results were unexpected, since hypophysectomized *F. heteroclitus* (Matthews, 1933; Abramowitz, 1937), as well as similarly operated eels (Fontaine *et al.*, 1949; Callamand *et al.*, 1951), and certain strictly fresh water teleosts (*e.g.*, Abramowitz, 1937; Chavin, 1954) have been reported to survive in fresh water for considerable periods of time.

The problem of osmoregulation in teleostean fishes has been extensively studied and is reviewed by Baldwin (1937), Krogh (1939), Black (1951) and Fontaine (1953). Despite much research, a hormonal control of water and salt balance, such as is known in higher vertebrates, has not been conclusively demonstrated in teleosts. In the mammal, pituitary control of osmoregulation involves at least two antagonistic renal functions: the neurohypophyseal antidiuretic factor (vasopressin) acting directly on the kidney tubules (reviewed by Gaunt *et al.*, 1951) and the growth hormone (GH) acting apparently in conjunction with corticotropin (ACTH) and thyrotropin (TSH) through the adrenal cortex, to increase urine flow (reviewed by White, 1955). In amphibians the neurohypophysis regulates water metabolism by both renal and extrarenal mechanisms. Injections of neurohypophyseal hormones promote glomerular antidiuresis, and resorption of water by the kidney tubules and bladder (Sawyer, 1956). Water uptake, which takes place primarily through the skin, is increased by the injection of neurohypophyseal hormones. This response is diminished after hypophysectomy but may be restored to normal by a combination of ACTH and TSH (Levinsky and Sawyer, 1952).

Mammalian neurohypophyseal hormones appear to have no effect upon teleostean osmoregulation (Keys and Bateman, 1932; Burgess, Harvey and Marshall, 1933; Boyd and Dingwall, 1939; Fontaine and Raffy, 1950). Salmon posterior lobe is also ineffective (Callamand *et al.*, 1951). Nevertheless, Arvy *et al.* (1954, 1955) observed that there was a depletion of neurosecretion in the hypothalamo-hypophyseal system of *Phoxinus* and *Anguilla* after transfer to hypertonic media. In mammals it is well known that an increased salt load leads to a depletion of neurosecretory material, correlated with a decrease in the content of antidiuretic

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hormone in the hypothalamo-hypophyseal system (see Bargmann, 1954). The secretion of neurohypophyseal hormones in mammals apparently also stimulates the rapid release of corticotropin (Martini and Morpurgo, 1955) and thyrotropin (D'Angelo, 1955) in response to stress. Therefore the depletion following salt stress in fish does not necessarily signify an antidiuretic function of the neurohypophyseal hormone(s).

However, there is some evidence of hormonal control of water-electrolyte regulation in fish. Kalashnikov and Skadovskii (1948) observed lowered blood chloride and osmotic pressure following injections of hypophyseal extracts in the sturgeon. Smith (1956) found that salinity tolerance was increased in trout by treatment with growth hormone. Many investigators have presented evidence favoring greater thyroid activity in hypotonic than in hypertonic media in a number of teleostean species; this was recently demonstrated, for example, in *Fundulus* by Gorbman and Berg (1955), using radioactive iodine uptake studies. Fontaine (1956) notes that survival of a number of marine teleosts in fresh water may be significantly prolonged by administration of thyroxin or TSH before transfer. Chavin (1954) found a transitory stimulation of the adrenal cortical tissue of goldfish following saline immersion which he attributed to release of ACTH. Keys and Bateman (1932) showed that adrenalin enhances chloride secretion in the eel gill, but the relation of adrenalin secretion to the pituitary is not obvious.

Regardless of the hormones which may be involved, three sites of salt and water balance in teleosts may be affected. Although fish lack the dermal water balance mechanism found in amphibia, hypophysectomy could affect the permeability of the water barrier, thereby having a profound effect upon survival in non-isotonic media. A second site is obviously the kidney, which is actively concerned in water elimination in fresh water fish. Henschel (1934–35) found that the euryhaline plaice, but not the stenohaline sole, was able to increase urine flow upon transfer to fresh water. But it must be noted that on the basis of morphological studies Grafflin (1937) concluded that the failure of fresh water teleosts to adapt to sea water or *vice versa* must be attributed to extrarenal factors.

The salt-regulating function of the gills is well known. Considerable attention has been given to the cytological aspects of this mechanism. Chloride excretion was first attributed to large, acidophilic cells in the gill epithelium by Keys and Wilmer (1932). These "chloride cells" were found to be absent in the dogfish but abundant in six marine species of teleosts. In four fresh water forms the cells were infrequent or absent although acidophilic cells lacking contact with both the surface and the basement membrane were noted. Mucous cells were more abundant in the latter group.

Liu (1942) demonstrated that an enormous development of "chloride cells," previously hardly distinguishable in the gill of the paradise fish, *Macropodus opercularis*, could be brought about by gradually increasing the salt concentration of its environment. Copeland (1948), investigating the "chloride cells" of *Fundulus*, found that they characteristically possessed an open, distal vesicle in the sea water-adapted state which was lost, a clear protoplasmic area being found in its place, when the fish were transferred to fresh water. The critical salinity separating the salt and fresh water pictures was thought to lie between $\frac{1}{16}$ sea water and $\frac{1}{100}$ cold-blooded Ringers solution in tap water (Copeland, 1950).

Getman (1950) found the same cell type in *Anguilla rostrata* with far less marked cytological variation between fish adapted to fresh or salt water. He found that the vesicles or "pits" were less frequent, not absent, in fresh water adaptation, but noted that the results might be attributable to experimental conditions. Hoar (1951) mentioned the development of "chloride cells" at the time of parr-smolt transformation in the Atlantic salmon preparatory to seaward migration. Nishida (1953) found that "acidophilic great cells," which he identified with the "chloride cells" of Hoar and Copeland, were much more abundant and better developed in the smolt of the sea-run than the land-locked variety of *Onchorynchus masou* while both were still in fresh water.

The present investigation was concerned with a number of endocrinological and histological aspects of the problem in *Fundulus*. After confirmation of Pickford's findings regarding the loss of ability of hypophysectomized sea water-adapted *F. heteroclitus* to live in fresh water, hypophysectomy of fresh water-adapted fish was used to determine whether the impairment is that of survival or adaptation. Replacement therapy was employed to verify the fact that the observed condition is due to pituitary loss rather than to another factor inherent in extirpation of the gland, and an attempt was made to identify the pituitary fraction which enables the animal to live. In investigating the cause of death, studies of weight and blood chloride changes were made. Lastly, an examination of the gill cytology of the fish was carried out in search of clearly defined histological differences which might help explain the osmoregulatory difficulties.

I wish to express my sincere appreciation to Dr. Grace E. Pickford for suggesting the problem and for her guidance and encouragement. For making available the facilities of the Bingham Oceanographic Laboratory for these investigations I owe my thanks to its director, Dr. Daniel Merriman. I am indebted to other members of the laboratory staff; to Dr. Gordon A. Riley for determining water salinities, to Mr. Edward C. Migdalski for supplying the perch, and to the Misses Harriet A. Chambers, Rita M. Eurich, and Patricia J. Harris for their technical assistance and advice. I also thank Dr. Sanford L. Steelman of Armour and Company for the ACTH, TSH, and posterior lobe preparation, Dr. A. E. Wilhelmi at the Department of Biochemistry, Emory University, for the purified growth hormone, Dr. Tillman D. Gerlough of E. R. Squibb and Sons for the thyroxin, and Dr. Richard H. Barnes of Sharp and Dohme for the desoxycorticosterone acetate. Facilities for collecting sea water were provided by Dr. Victor L. Loosanoff, director of the U. S. Fish and Wildlife Service Laboratory at Milford, Connecticut. Finally, for translations of the Russian and Japanese literature employed I express my appreciation to Dr. Alexander Petrunkevitch and Mr. Mikiso Hane, respectively.

Materials and Methods

1. Running fresh water system

In order to test a relatively large number of hypophysectomized fish and controls under satisfactory conditions a running fresh water system was required. Such a system was necessary to avoid possible adverse effects from the handling involved in daily water changes or the definitely undesirable salt accumulation which would occur

in a standing tank from addition of food, evaporation, etc. As the only fresh water readily available was from the municipal water supply, three problems presented themselves in designing the required system: constant rate of flow, constant temperature, and dechlorination.

The arrangement is shown in Figure 1. Tap water was received into a constant level tank (A) placed above the aquaria. The water flowed from the tank by gravity at a rate regulated by a clamp (B) through a series of ten five-gallon carboys (C 1–10). The first two contained activated charcoal in sausage skins (D 1 and 2) to remove dissolved chlorine (modified method of Coventry, Shelford and

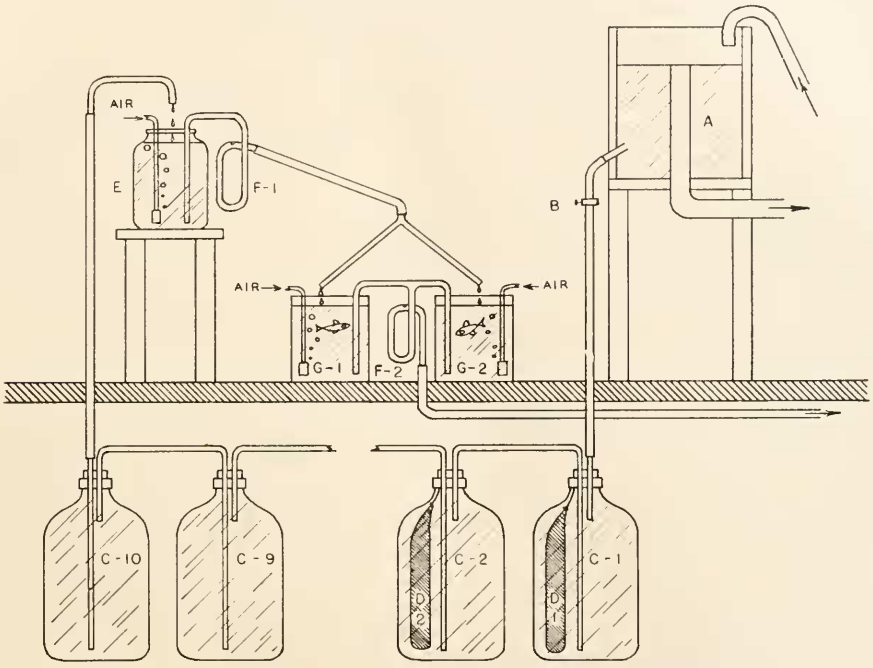

FIGURE 1. Running fresh water system (drawn by Patricia J. Harris).

Miller, 1935). The outflow from the last carboy was dripped into an aerated container (E) for the purpose of reoxygenation. From here water was fed into the aquaria (G 1 and 2) by means of a constant level siphon (F 1), a similar arrangement being used to adjust the outflow (F 2).

The flow of water was regulated to deliver ten gallons a day to each of two one-gallon aquaria, thereby giving a turnover rate of about once in every two and one-half hours. The battery of carboys created a reservoir such that it took over two days for water to pass through the system. During this time it reached aquarium room temperature which was regulated at *ca.* 15° C. Tests of the outflow with orthotolidine indicator showed it always to be chlorine-free. The only difficulty encountered was bubbles in the reservoir lines brought about by warming of the water. Flow irregularities due to these were easily avoided by removal of one of

the carboy stoppers for a few seconds once or twice daily. Five to seven fish could be maintained comfortably in each aquarium.

2. Experimental procedures

All fish used were *Fundulus heteroclitus* males and females 2¾ to 3¼ inches in length. Hypophysectomy, feeding, and illumination arrangements were the same as described by Pickford (1953). Operations and injections were made under anesthesia with tricaine methane sulfonate. Fresh rather than salt water was used exclusively when such procedures were being carried out with fish being maintained in the fresh water system. All experiments were made at 14–17° C. The pH of the sea water from the storage tank and in freshly diluted mixtures was *ca.* 8.5. However, in long established sea water tanks where the fish were usually held it fell to *ca.* 7.4. The pH of the fresh water ranged from 7.2 to 7.6.

One or two unoperated controls were kept in the aquaria with each experimental group, making a total of about 30 normal fish being maintained in fresh water for periods of two weeks to three months each. All of these fish remained healthy, although, as also in the case of fish in salt water, it was occasionally necessary to treat them for minor skin infections, either with aureomycin or malachite green as recommended by Pickford (1954a). Hypophysectomized fish in salt water rarely die as an immediate result of the operation but a small number of fish, of the order of 5–10%, were lost at later dates from causes other than the experimental treatments. In all a total of about 100 hypophysectomized fish were employed in the experiments.

(*a*) *Tests on survival of "long term" hypophysectomized sea water-adapted fish in fresh water.* A group of about 60 fish that had been kept in sea water were hypophysectomized and returned to this medium. They were allowed to recover for at least two weeks and were then tested for their ability to survive in fresh water. Out of 56 so tested only three were able to live indefinitely, and these showed positive evidence of incomplete hypophysectomy (growth in length and, in males, development of nuptial coloration); they were therefore excluded from the experiment. The remaining 53 fish developed symptoms of asthenia, described below, but 39 of them were saved by gradual return to sea water through 1/10, ¼, and ½ dilutions over a period of about 24 hours. Fish that recovered were kept under observation until they were again eating normally and then were allowed at least a few extra days before being used in other experiments. The average time for complete recovery was about two weeks.

(*b*) *Tests on survival of "long term" hypophysectomized sea water-adapted fish in diluted sea water.* These experiments were limited to a small number of previously untested fish and are of a preliminary nature. The tests were carried out in similar aquaria to those used in the fresh water system, the water being changed two or three times weekly. Two hypophysectomized fish and one unoperated control were tested in 1/10 and ¼ sea water (*ca.* 2.6 and 6.5 ‰, respectively); four hypophysectomized fish and one control were tested in ½ sea water (*ca.* 13 ‰).

(*c*) *Tests on ability of fresh water-adapted fish to survive hypophysectomy in fresh water.* A group of 25 fish were kept in fresh water for about four weeks before operation and were returned to this medium after removal of the pituitary. Survival times were studied as in the preceding groups and those that recovered in sea water were saved for other experiments.

(*d*) *Tests on ability of sea water-adapted fish to survive transfer to fresh water immediately after hypophysectomy.* A small group of eight sea water-adapted fish were transferred to fresh water immediately after hypophysectomy. These served as controls for the group that had been pre-adapted to fresh water.

(*e*) *Weight changes in fresh and salt water.* Two series of experiments were made in each medium with four previously untested, hypophysectomized fish in each group. One series was weighed daily until onset of asthenia in the fresh water group and for a comparable time in the sea water group. A second series was treated similarly but received only initial and final weighings. Two unoperated controls were included with each group, except those receiving initial and final weighings in sea water, making a total of 22 fish employed in the experiment. Weighings were made on a triple beam balance following anesthesia and extremely light blotting. It is realized that the small numbers employed detract from the significance of this experiment but time did not permit further study and the results appear to be worth reporting.

(*f*) *Blood chloride determinations.* Blood chlorides were determined by a microadaptation of the method of Schales and Schales (Hawk, Oser and Summerson, 1947). Blood serum samples were collected from normal fish adapted to salt or fresh water (8 days) and from hypophysectomized fish adapted to sea water and on the verge of death in fresh water (4–7 days). Whole blood was obtained by cutting off the tail and allowing the blood to drop on to a paraffined spot plate. Ten- to 30-mm.^{3} samples were centrifuged in an air driven "spinning top" rotor (Glick, 1949) and 4.8-mm.^{3} quota of serum were transferred into titration vessels with the aid of a constriction pipette. Before titration two magnetic beads and *ca.* 45 mm.^{3} of water containing 0.06 ml. of indicator solution per 2 ml. were added to the sample. Titration was made without deproteinization using a 70-mm.^{3} microburette and magnetic stirrer.

(*g*) *Hormone therapy.* These experiments were all made on hypophysectomized fish that had been previously tested for their inability to survive in fresh water and had subsequently been allowed to recover in sea water.

Fourteen series of intraperitoneal injections were made in an attempt to determine what treatment would enable hypophysectomized *Fundulus* to survive in fresh water. Breis of *F. heteroclitus, Pollachius virens,* and *Perca flavescens* pituitaries, in addition to solutions or suspensions of ACTH, desoxycorticosterone acetate (DOCA), TSH, thyroxin, GH, posterior lobe preparation, a combination of ACTH, TSH, and GH, and three series of saline controls, were employed. The breis and hormones were made up in 0.6% NaCl. Unless otherwise noted, groups of four fish were given daily injections of 0.005 ml. per gram weight on alternate days with four injections (one week) in sea water before transfer to fresh water.

Fundulus pituitary glands were collected from fish weighing from seven to 40 grams, caught near New Haven just prior to spawning in April, 1955. Two hundred glands, averaging slightly under one milligram each in weight, were contained in each milliliter of homogenate so that each fish received one gland per gram weight at each injection. Fresh frozen glands from the stenohaline marine pollack were collected from prespawning fish during the summer of 1954, at Wilson's Beach, Campobello Island, by G. E. Pickford and assistants. The brei was prepared in about the same concentration by weight as the *Fundulus* material, 200 mg./ml. Two series of experiments were carried out using this preparation. The first was

under the same conditions as with the *Fundulus* brei. The second series, comprising two hypophysectomized fish and one unoperated control, was given daily treatment with the homogenate at 0.01 ml. per gram and with but two injections before transfer to fresh water. Thus they received four times as much material as did the first series. The average weight of the pituitary of post-spawning yellow perch weighing from 30 to 100 grams each was found to be of the same order as that of pre-spawning *Fundulus* weighing 7–40 grams, *ca.* 1 mg., and hence the same concentration (200 glands/ml.) was used. Post-spawning fish, caught during June–July, 1955, in the New Haven area, were employed.

ACTH dosage (Armour's Acthar Lot No. R-491284-U, 0.05 USP units/gm.) was based on that used by Pickford (personal communication) who obtained definite stimulation (increased cell layers, proliferation) of the interrenals of *F. heteroclitus* within one week. Desoxycorticosterone acetate injections (Sharp and Dohme Lot HXVII-06-07-4-1, 25 μg/gm.) were equivalent to those given by Kirshenblatt (1952) to vy'un (*Misgurnus fossilis*) in which he was able to induce egg maturation and ovulation. On the basis of the thyroid stimulation findings of Pickford (1954b) 1 μg/gm. was selected for injections of TSH (Armour Lot No. 317–51, 1.2 USP/mg.). About 5 mm.^{3} 0.1 N NaOH/ml. was required to bring this into solution. Since there is no established function in fish on which to base dosage, thyroxin (Squibb BT00080) was somewhat arbitrarily set at 2 μg per gram weight. It is of the same order as that used by many investigators on fish and has been shown to increase resistance to skin infections in *Fundulus* (Pickford, unpublished observations). Addition of 20 mm.^{3} 0.1 N NaOH/ml. was required to dissolve the hormone. In these two series, which were made early in the investigation, before the effects of *Fundulus* brei had been established, only two injections in sea water prior to fresh water transfer were made and injections were given daily.

Growth hormone dosage (Wilhelmi Beef B168GH, 10 μg/gm.) was based on that given by Pickford (1954a) by which she obtained growth of the same order as occurs in normal fish. Ten mm.^{3} 0.1 N NaOH/ml. were required for solution. Injections of whole posterior lobe preparation (Armour Hog Lot No. 503, 100 mU/gm.) were given at twice the dosage determined as one *Fundulus* unit in spawning reflex assays (Wilhelmi, Pickford and Sawyer, 1955). The concentration of each hormone in the GH-TSH-ACTH combination remained the same as when used separately: 10 USP units ACTH, 2 mg. TSH (Armour Lot No. 2R3, 0.4 USP/mg., less potent then 317–51 but still at a higher dosage than that used effectively by Pickford, 1954b), 10 mg. GH, 15 mm.^{3} 0.1 N NaOH/ml. Of three series of saline controls, the first received injections of 0.6% NaCl under the usual conditions. A second and a third group, each with two hypophysectomized fish and an unoperated control, were injected daily with 0.6% and 0.06% NaCl. No build-up injections in sea water were made with these last two series.

(*h*) *Histological study.* The thyroids and the interrenals of the group receiving *Fundulus* brei were treated as previously described by Pickford (1953). Hemoglobin was determined with the aid of a Tallqvist-Adams scale. The study of the gill cytology was carried out according to the Regaud-Altmann mitochondrial technique recommended by Copeland (1948). Experience proved that best results could be obtained if the picric acid differentiating fluid were used merely to wash off excess acid fuchsin after cooling and differentiation were accomplished by flooding the slide with 1% aqueous methyl green for about 5–10 seconds. It is worth

noting that the heating of Altmann-flooded slides should be stopped when white fumes are first evoked.

Gills of four normal sea water-adapted *Fundulus,* five normal fresh water-adapted specimens, six hypophysectomized fish which were sea water-adapted, four hypophysectomized *F. heteroclitus* on the point of death in fresh water, and those of the series receiving *Fundulus* brei were selected for study. Each set was also stained with hematoxylin and eosin and by the azan method for confirmatory studies.

Results

(*a*). Of the total of 53 long-term hypophysectomized fish tested by transfer to fresh water, 14 were found dead or so nearly so as not to recover subsequently.

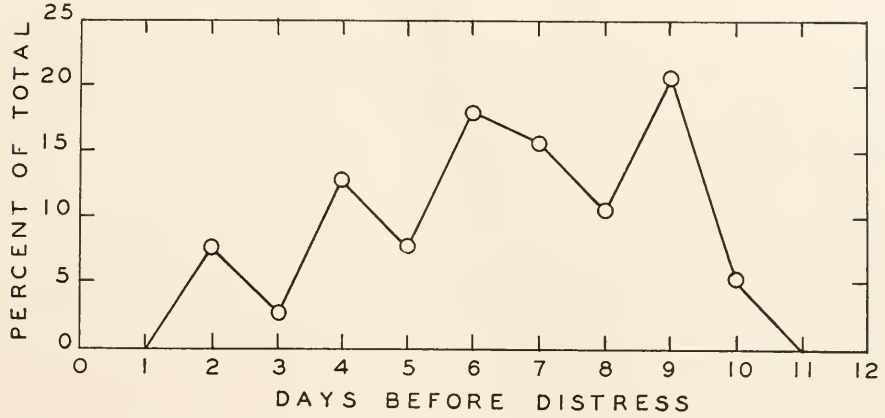

Figure 2. Time of onset of asthenia in 39 sea water-adapted "long term" hypophysectomized *F. heteroclitus* after transfer to fresh water. Only fish that were saved and recovered in sea water are used (see text).

The 39 remaining fish, removed when found lying on their sides, recovered completely after being transferred to sea water. These latter fish and the number of days they remained in fresh water before removal was necessary, are shown graphically in Figure 2. Only these 39 are considered since it is felt that subsequent recovery in sea water is the most conclusive demonstration that the fresh water was the sole cause of their distress.

The following symptoms preceded death in fresh water: The fish refused to eat after the first or, occasionally, the second day. After about two thirds of the total time which elapsed before death they became extremely quiescent and stayed on the bottom of the aquarium. About a day before death the fish were noticeably tilted and would be found lying completely on one side twelve hours later, usually ventilating their gills rather rapidly. This was the point at which there could be no doubt that the fish were succumbing and this stage was selected for rescue for use in subsequent experiments. Shortly before death they would be found completely upside down and frequently could be saved even in this state. Return to sea water was accompanied by hydrostatic and equilibrium difficulties far exceeding those experienced by normal fish.

TABLE I

Time of onset of severe asthenia in "long term" hypophysectomized, sea water-adapted F. heteroclitus in different dilutions of sea water

Per cent sea water	Number of fish	Days				
		5–10	11–15	16–20	21–25	26–30
10%	2	—	2	—	—	—
25	2	—	—	2	—	—
50	4	1	—	1	—	2

Time did not permit study of the ability of these fish to withstand a second transfer to fresh water, since the tested fish were needed for hormone therapy. Such a project would have required the study of the entire group, in order that possible differences in mean survival time would be statistically significant, and this would have greatly curtailed the study of other aspects of the problem. For replacement therapy experiments it appeared more important to use saline-injected controls since it is known that such treatment tends to increase the weight loss and lower the viability of hypophysectomized *F. heteroclitus* in salt water.

(*b*). Table I summarizes the results for fish tested in diluted sea water. At all three dilutions the normal controls were still active and eating long after their hypophysectomized counterparts had succumbed.

(*c and d*). The results obtained by operating on fresh water-adapted fish and placing sea water-adapted fish in fresh water immediately after the operation are graphed in Figure 3. Owing to the small number of cases in these two groups,

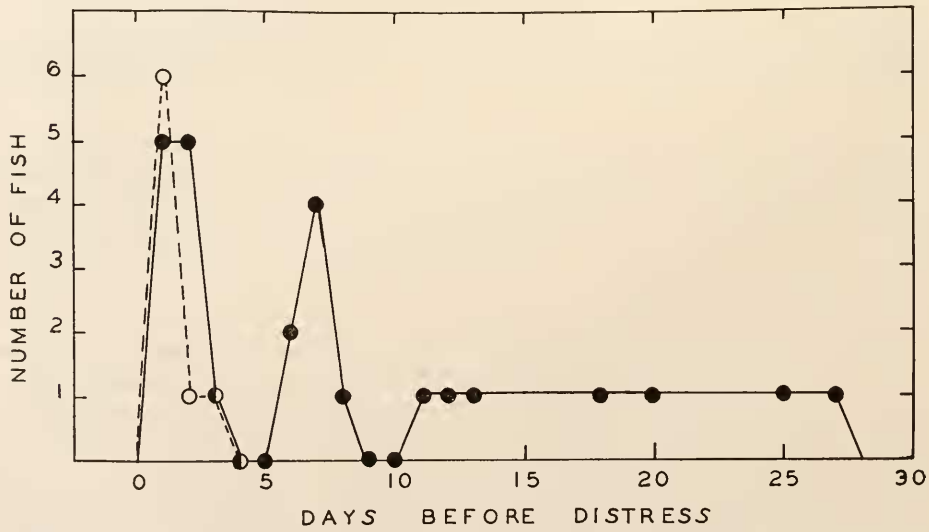

FIGURE 3. Time of onset of asthenia or death in hypophysectomized *F. heteroclitus* placed in fresh water immediately following the operation. Solid line represents 25 fish previously fresh water-adapted. Broken line indicates the eight previously sea water-adapted.

TABLE II

Weight change tests

Group		Days	No. of fish	Mean weight change	*s*
Daily weighings:					
Fresh water	Hypects	2–3	4	13.8	3.4
	Controls	2	2	−0.1	—
Sea water	Hypects	3	4	−4.0	3.2
	Controls	3	2	−6.5	—
Two weighings only:					
Fresh water	Hypects	6–9	4	4.3	4.5
	Controls	10	2	7.0	—
Sea water	Hypects	6	4	−4.1	5.0

those which did not recover subsequently are included as well. Out of the group of 25 fresh water-adapted fish, 11 succumbed during the first three days, seven survived 6–8 days, and another seven survived 11–27 days. In the second group, all died in the first three days, six after one day, and one on each of the following two days.

(*e*). Table II contains the results of the weight change tests. Hypophysectomized fish succumbed more rapidly in fresh water when handled daily than when weighed only at the beginning and end of the experiment, 2–3 days as compared with 6–9 for the latter group. Both hypophysectomized groups showed a slight weight increment at the time of onset of asthenia in fresh water, greater in the case of daily handling (13.8% versus 4.3%). Hypophysectomized fish in sea water showed a small decline (*ca.* 4%), irrespective of whether they were handled daily or not. The number of controls is too small in each group, but appears to indicate that daily handling causes weight loss in sea water but little change in fresh water. A mean weight increment in unhandled fish in fresh water is probably due to natural growth, since a period of 10 days elapsed.

(*f*). In Table III blood chloride determinations are given. Normal fish in either sea water or fresh water maintain a constant blood chloride level (0.885–0.887) and the blood chloride of hypophysectomized fish in sea water is not sig-

TABLE III

Blood chloride determinations

Group	Number of fish	Serum NaCl, gm.%		
		Range	Mean	*s*
F.W. Hypects	6*	.322–.451	.383	.049
F.W. Normals	6	.809–.996	.887	.078
S.W. Hypects	5	.777–.872	.817	.036
S.W. Normals	5	.818–.957	.885	.051

* Samples were taken at the onset of acute symptoms. Three of the fish in this group had been receiving GH-TSH-ACTH and DOCA injections.

nificantly lower ($P = 0.05$–0.1). When hypophysectomized fish are placed in fresh water, however, a blood chloride averaging only 43% of normal is found when asthenia begins.

(g). The results of injection therapy are found in Table IV. The fish's numbers are given so that it can be seen which ones were used in more than one series and their performance in each compared. Those receiving *Fundulus* brei were still active and eating after 20 days in fresh water. At that time it was decided that the preparation was undoubtedly enabling them to survive, so the fish were killed for autopsy. Numerous fish being tested for fresh water survival for the first time or receiving injections of other preparations succumbed during this 20-day period.

The results of the autopsy of the fish receiving *Fundulus* brei (Table V) indi-

TABLE IV

Results of injection therapy

Material injected	Fish number	Days in fresh water		Subsequent recovery completed (days)
		Previous test	With injections	
Fundulus brei†	4	7	20	(Discontinued)
	25	(*)	20	(Discontinued)
	55	6	20	(Discontinued)
	107	9	20	(Discontinued)
Perch brei†	68	10	6	Died next day
	58	8	8	3
	B108	(*)	16	Found dead
	G3	6	19	(Discontinued)
Pollock brei (Alternate days)	14	6	2	5
	77	9	3	3
	68	10	5	4
	58	8	7	3
Pollock brei (Daily)	12	4	4	Died 3 days later
	11	3	6	Died 2 days later
ACTH	76	6	3	Found dead
	44	(*)	5	4
	78	7	5	3
	32	(*)	5	3
DOCA	44	(*)	2	Found dead
	14	6	2	2
	61	7	2	Died 16 days later
	32	(*)	4	Found dead
	G25	10	5	Blood sample taken

* Fish tested by use in weight change test or hypophysectomized after fresh water adaptation and hence not comparable.

† In each of these groups a fifth fish died under anesthesia after the fourth (perch) or fifth (*Fundulus*) injection. These are most unusual circumstances not otherwise encountered in many hundreds of injections.

TABLE IV—*Continued*

Material injected	Fish number	Days in fresh water		Subsequent recovery completed (days)
		Previous test	With injections	
TSH	21	6	3	Died next day
	18	(*)	4	6
	17	(*)	5	6
	24	6	5	4
Thyroxin	4	7	3	13
	26	5	3	16
	9	6	5	Died 20 days later
	2	4	6	5
GH	44	(*)	4	4
	77	9	4	4
	G3	6	5	5
	68	10	5	4
GH-TSH-ACTH	G13	9	2	Found dead
	G29	9	3	Found dead
	G26	5	3	3
	G30	7	4	Blood sample taken
	46	3	4	Blood sample taken
Posterior lobe preparation	32	(*)	3	4
	14	6	4	5
	78	7	4	4
	G23	7	9	Died next day
0.6% NaCl (Alternate days)	47	4	2	Found dead
	G12	5	3	Found dead
	96	8	2	Died 8 days later
	46	3	5	5
0.6% NaCl (Daily)	11	3	2	3
	9	6	4	6
0.06% NaCl (Daily)	8	4	3	10
	2	4	4	5

cate moderate to strong stimulation of the thyroid and interrenal. Sexual maturation was nearly as great as in normal prespawning *Fundulus*. The experiment was of too short duration to evaluate growth, but length increments in two of the fish indicate the presence of growth hormone, despite the small average weight loss, probably attributable to multiple effects of treatment. The anemia of hypophysectomy was not alleviated.

Injections of pollock brei failed to demonstrate any salutary effect. Nuptial colors, indicating the presence of gonadotropins, appeared in some males during the 2–7 day period before onset of acute symptoms, but since test of subsequent recovery in sea water seemed important, autopsy was not performed to check the presence

of other hormones. Due to time limitations the experiment employing the perch pituitary brei was discontinued before the full 20 days, although one fish was still showing none of the symptoms preceding death in fresh water at that time.

The daily pollock brei treatment given to a normal fish in fresh water over a twelve-day period gave evidence of no adverse effects. The saline controls were also unaffected by the injections received. Yet, except for the perch and *Fundulus* breis, none of the injections gave indications of increasing the ability of the hypophysectomized fish to survive in fresh water.

TABLE V

Condition of fish receiving Fundulus brei at the time of autopsy compared with normal values. (GSI = Gonad Wt. × 100/Body Wt.; HSI = Liver Wt. × 100/Body Wt.; TEH = Average thyroid cell height in microns)

Fish number and sex	Wt. change %	Length change %	GSI	HSI	Anterior interrenal	TEH	Hemoglobin
Fundulus brei:							
4 ♂	− 2.1	−0.8	3.9	3.9	Hypertrophy	5.0	45
55 ♂	−10.1	1.4	6.9	5.1	Hypertrophy	14.4	55
107 ♂	1.5	0	3.1	4.8	Hypertrophy	12.4	50
25 ♀	− 1.3	1.7	4.4	5.2	Hypertrophy	6.0	45
Hypect controls:*							
♂♂	− 1.2	−0.2	0.3	5.4	Inactive but not atrophied	3.8	68
♀♀	−10.4	0	1.1	6.4	Inactive but not atrophied	—	54
Normal wild fish:**							
♂♂ Nov.	—	—	0.7	6.3	—	6.5	—
May	—	—	5.8	2.8	—	6.9	80
♀♀ Nov.	—	—	1.5	6.4	—	6.0	—
May	—	—	6.1	4.8	—	5.9	—

* Males (Pickford, 1954b), means of five fish saline-injected thrice weekly for one month in sea water. Females (Pickford, unpublished) means of three saline-injected daily for one week in sea water.

** Means of about 40 fish per group ♂♂ (Pickford, 1953), ♀♀ (Pickford, unpublished), taken from sea water.

(*h*). In the histological study, the sea water-adapted fish were found to have a gill cytology essentially as described by Copeland (1948): the chloride cells were columnar, acidophilic, reaching from the free surface to the basement membrane, and usually with an "excretory vesicle" at the distal border. In normal fish adapted to fresh water for a week or more, however, most of the cells exhibited shrinkage, rounding up, and frequent loss of contact with the free surface; they were also far less fuchsinophilic. Typical sea water-adapted cells, sometimes with vesicles, were noted, but less commonly. In hypophysectomized fish in fresh or sea water the chloride cells were cytologically the same as normal fish in these respective media.

A cursory count of the mucous cells revealed an average of about four per section in the sea water-adapted and eight in the fresh water-adapted normal fish.

The mucous cells of hypophysectomized fish were emptied and atrophied in contrast to the normal condition. In addition, although the sea water-adapted fish had about four mucous cells per filament section, as did their unoperated counterparts, in hypophysectomized fish in fresh water the number was about two per section, much less than normal. Hypophysectomized fish receiving *Fundulus* brei in fresh water showed the unoperated fresh water-adapted picture; the mucous cells appeared full, well stained, and averaged seven to eight per section.

Discussion

1. Survival in fresh water

There is no doubt that untreated hypophysectomized *Fundulus heteroclitus* cannot withstand transfer to fresh water under the conditions of these experiments. Many fish were saved and used again for unsuccessful injection therapy, injections which apparently have no adverse effects upon hypophysectomized fish in sea water or normal fish in either medium. Adding these 46 instances in which the fish were retested and still failed to survive to the 53 of the first test, a total of 99 cases are available to support this thesis. The more than thirty normal fish, unaffected by transfer from salt to fresh water at various times in company with the experimentals, exclude the possibility of harmful factors in the running fresh water system.

It would seem from the survival time (Fig. 2), varying between two and 10 days with an average of about 6½ days, that the cause of distress is slow in reaching its full effects. The form of the curve, rising slowly and dropping suddenly, supports this contention. It is highly unlikely that the irregularity of the curves derives from any other cause than lack of sufficient numbers to smooth it out.

The wide range of survival times obtained by operating on fresh water-adapted fish appears to show three asthenia peaks (Fig. 3). Out of the group of 25 fish, 44% succumbed during the first three days, 28% survived 6–8 days, and another 28% survived for 11–27 days. These results may be compared with the incidence of asthenia in sea water-adapted fish transferred to fresh water two or more weeks after hypophysectomy (Fig. 2). In this group there was a single peak at 6–7 days, only 10.3% died during the first three days, and none survived longer than 10 days. On the other hand, when sea water-adapted fish are transferred to fresh water immediately after removal of the pituitary they succumb rapidly (Fig. 3, broken line); out of a group of eight such fish none survived more than three days, although it is extremely rare for hypophysectomized fish to die (except later, from collateral effects such as the development of kidney stones, Pickford, 1953) when they are returned to sea water. The survival time in fresh water-adapted *Fundulus* therefore appears to be the result of two opposing factors: an increased sensitivity to fresh water immediately after the operation, and, if this period is withstood, a tendency for the prolongation of survival which could be attributed to pre-existing fresh water adaptation.

The results of Abramowitz (1937) and Matthews (1933), referred to in the introduction, remain unexplained. But the survival time which they noted ("several weeks") is not far outside the limit for fish hypophysectomized in fresh water reported herein. Even with normal *Fundulus* there has been a discrepancy between the results of different authors (Garrey, 1905; Sumner, 1906).

2. Cause of death

The low blood chlorides of hypophysectomized fish dying in fresh water (Table IV), less than half of that of operated fish in sea water or normal fish in either medium, is seemingly sufficient cause for death. These studies indicate that *Fundulus* normally has remarkable osmoregularity capacity, no difference being discernible in the blood chlorides of fresh and salt water-adapted fish. Rather than having a slightly higher blood chloride, resulting from decreased osmoregulatory capacity, the hypophysectomized fish in sea water appears to have a somewhat lower blood chloride although the difference is not quite statistically significant ($P = 0.05$–0.1).

Examples of serum chloride determinations on various fish are presented for comparison in Table VI. It is well known that the osmotic pressure of the blood of stenohaline marine teleosts is higher than that of fresh water species, and that euryhaline species are capable of a considerable degree of regulation. The change in blood chlorides is even greater than the fall in osmotic pressure accompanying

TABLE VI

Comparative data on blood chloride for fresh water, sea water and euryhaline teleosts

Author	Species	Serum or plasma NaCl in gm. per cent	
		F.W.	S.W.
Strictly fresh water:			
Vars (1934)	*Cyprinus carpio*	av. 0.477	—
	Esox lucius	av. 0.487	—
Puschel (1928)	*Tinca tinca*	av. 0.517	—
Drilhon (1942)	*Cyprinus carpio*	av. 0.626	—
Stenohaline marine:			
Hall, Grey, and Lepkovsky (1926)	*Brevoortia tyrannus*	—	0.862
Grafflin (1935)	13 species	—	0.875–1.008
Boucher-Firly (1935)	*Conger conger*	—	0.994–1.097
	Muraena helena	—	1.035–1.228
Euryhaline poikilo-osmotic:‡			
Boucher-Firly (1935),	*Anguilla anguilla*		
Callamand (1943), and	immature	0.375–0.701	0.661–1.170
Callamand *et al.* (1951)	hypophysectomized	—	0.631–0.769
	silver, migrating	0.155–0.491*	0.826–1.338
Euryhaline homio-osmotic:			
Koch and Heuts (1942)	*Gasterosteus aculeatus*		
	immature**	0.490–0.650	0.510–0.620
Present paper	*Fundulus heteroclitus*		
	normal	av. 0.887	av. 0.885
	hypophysectomized	av. 0.385†	av. 0.817

* Asthenia is associated with hypochloremia. In a later paper (Fontaine and Callamand, 1948) higher values for some silver eels are given.

** Sexually mature sticklebacks lose their ability to withstand higher salinities.

† Asthenic and on the verge of death.

‡ Osmotic pressure data, summarized by Black (1951) and Kalashnikov (1939), indicate that *Salmo trutta*, *S. salar* and *Acipenser stellatus* belong in this group.

transfer to lower salinities because of the concurrent increase in alkaline reserve (Fontaine and Boucher-Firly, 1934). Two types of euryhalinity are found in fish. In the first, exemplified by the eel, the blood chloride may fluctuate considerably with the environment, whereas in the stickleback and killifish it remains constant. It will be noted that in the hypophysectomized eel, as well as *Fundulus,* the blood chloride of hypophysectomized fish in sea water tends to be lower than normal. The generally lower blood chloride of *Anguilla* in fresh water is undoubtedly related to the fact that this fish is not capable of absorbing chlorides through the gills.

The proportionately small weight changes (Table III) indicate that the salt-regulating mechanism rather than failure of ability to excrete excess water is at fault. In addition, the fish showed no signs of edema such as would result from internal flooding.

The results of the tests in diluted sea water are surprising. The salinities of the $\frac{1}{10}$ and $\frac{1}{4}$ dilutions are below that of *Fundulus* blood. The results show, as might be expected, that survival is merely prolonged. The half and half dilution, equivalent to 1.30 gm. per cent NaCl, is considerably above the salinity of the blood, yet the fish did not survive indefinitely in this medium. The present investigation throws no light on the problem posed by this evidence.

The incidence of renal calculi was not studied. This trouble develops slowly over a period of three weeks or longer and is sporadic (Pickford, 1953). It can be excluded as a cause of death in fresh water since fish operated in fresh water also failed to survive long enough (except in a few instances) for renal lethiasis to develop. One of the four receiving *Fundulus* brei (No. 25) had calculi, seen in sections of the head kidney, yet survived as well as the rest.

3. Hormone therapy

Although there were only four cases, owing to limitations set by the difficulties involved in obtaining a large supply of *Fundulus* glands, it is clear that these injections were responsible for the long survival of the fish in fresh water. The experiment seems to have been adequately controlled to warrant acceptance of this fact: 1) the fish were pretested, 2) they were allowed to stay in fresh water twice as long as the maximum survival time for non-injected sea water-hypophysectomized fish and were still in good health at the end of that time, 3) controls receiving saline under identical conditions showed no increased survivability, and 4) untreated hypophysectomized fish kept in the aquarium at the same time succumbed as usual. It therefore appears safe to conclude that the *Fundulus* pituitary secretes a hormone or hormones enabling the fish to survive in fresh water.

Since all target organs of the adenohypophysis appear to have been stimulated by the *Fundulus* brei, the evidence from the autopsy throws no light on the nature of the particular factor involved.

Since two of the fish receiving perch pituitary brei survived in fresh water considerably longer than the established maximum for hypophysectomized *Fundulus,* it also seems most probable that the factor enabling the fish to live was present in this preparation. The fact that perch brei was not as successful as the *Fundulus* brei could be due to suboptimal amounts of the factor, wrong proportions in the event that more than one hormone is involved, or adverse foreign protein reactions.

Pollock pituitary, whether given under the same conditions or in quadruple

amounts (double dosage daily), did not aid survival. This preparation, therefore, either lacks the necessary factor altogether or one of the adverse factors mentioned as possibly present in the perch brei holds an even greater sway in the pollack preparation.

There is every indication that ACTH, TSH, GH, posterior lobe preparation, DOCA, and thyroxin alone or the first three in combination have no salutatory effect. Again it is possible that the dosages were too high or too low, but those of the first four were known to have a moderate influence on target organs. The conditions of injection are even less likely to be at fault. Most were given exactly as the successful *Fundulus* brei. Daily injections may be disturbing since daily weight change tests, not reported here, indicated that such handling was highly undesirable. However, in the presence of the necessary factor for survival it would seem that the hypophysectomized fish could overcome this difficulty, since daily handling does not seriously disturb normal fish in fresh water. Moreover, daily injections would seem essential for thyroxin, which presumably does not act through a target organ. The negative results with all these preparations are surprising in view of the literature which implicates one or the other in teleostean osmoregulation. The results with the posterior lobe preparation serve to substantiate previous findings—that these hormones apparently have no osmoregulatory effect in fish.

All the possibilities have not been exhausted in the tests with these hormones. Dosage levels have not been explored. Three well known vertebrate hormones, prolactin, gonadotropin, and intermedin, remain untested. There is no reason to suspect intermedin, which undoubtedly was present as a contaminant in many of the preparations tested. Gonadotropins also seem unlikely since immature killifish withstand transfer to fresh water as well as mature fish of either sex. In addition, both the pollock and perch breis contained relatively high amounts of the gonad-stimulating hormone and only one of these was successful, whereas the perch brei, evidently with low gonadotropic activity, had a salutatory effect. Prolactin has no known function in fish. Although an osmoregulatory control would not be suspected of the hormone from its known activity in higher vertebrates, it is not ruled out. More likely, however, since the pituitary breis of *Fundulus* and the strictly fresh water perch possess the factor, whereas it is probably absent in the marine pollock, an as yet undescribed hormone is involved.

4. Gill cytology

The cytological picture of the gills of normal *Fundulus* adapted to sea water was found to be exactly as that described by Copeland (1948) and requires no further comment. The results for fresh water adaptation obtained in this study more closely resemble the condition described by Copeland for fish in distilled water than his description of the condition in tap water. The discrepancy could be due to differences in the calcium content of the water or a similar cause which could be brought out only by complete tap water analyses. There is no doubt that the chloride cells are shrunken and less strongly fuchsinophilic in fresh water. When gills of fish adapted to each of the two media were stained on the same slide by the Altmann method, the chloride cells of the fresh water specimen were scarcely to be seen, while the sea water sections were excellent.

The fresh water cytological picture noted in this study is not unlike that described by Keys and Wilmer (1932), who found the chloride-secreting cell-type less abundant or absent in fresh water fish as compared with marine forms, while some acidophils were also seen rounded up below the epithelium in the former. The findings herein are also reminiscent of the hardly distinguishable cell in *Macropodus* before saline adaptation (Liu, 1942), the merely lessened abundance of chloride cells with vesicle noted by Getman (1950) in the eel, and the lesser chloride cell development noted by Nishida (1953) in the land-locked as compared with the sea-run salmon.

The fact that the chloride cells appear normal in hypophysectomized sea water-adapted fish is in keeping with the chloride excretion function, long attributed to these cells, since blood-chloride studies indicate that the fish maintain osmotic equilibrium perfectly well in this hypertonic environment. The obvious change in these cells after transfer to fresh water in normal fish was again found in hypophysectomized ones, indicating normal response as far as cutting down of chloride excretion is concerned.

Mucous cells are apparently holocrine. These are the only cells in the mammalian gastric mucosa which have a significant mitotic rate, presumably to replace cells extruded into the cavity of the gut (Stevens and Leblond, 1953). However, observations on the digestive system of hypophysectomized rats (Baker and Abrams, 1955) showed that the mucous cells are not affected. Nevertheless it appears that those in the gills of hypophysectomized *Fundulus* have discharged their contents and have not been replaced. Mucous cells may play a special role in fresh water fish. They were noted to be more predominant in the gills of fresh than in sea water-adapted fish in this investigation, and Keys and Wilmer found them so in fresh water over marine teleosts. The skin was not studied in the present investigation, but it appears likely that all epidermal mucus secretion would be under pituitary control if such is the case in the gills.

The protective role of mucus in the osmoregulation of fishes is well known. Firly (1932) found that removal of mucus from the skin by washing was more harmful to eels transferred from fresh to salt water than *vice versa*. The opposite effect was observed in this study; however, isotonic and hypotonic media were found to be deleterious, whereas the fish apparently maintain themselves well in hypertonic sea water. It appears obvious from the weight change and blood chloride studies that hypophysectomized *F. heteroclitus* in fresh water are able to cope with the increased water load imposed by the medium but suffer from an inability to maintain the normal blood chloride level. Since the site of chloride loss, whether through the gills, skin, or kidney is unknown, it is not possible to state definitely that atrophy of the mucous cells is involved. An increased permeability of the skin, resulting from lack of a protective coating, would lead to polyuria (Grafflin, 1931) with possible abnormal loss of chloride. But the failure to survive, even in an approximately isotonic medium, indicates that other factors are operative.

Summary

1. Hypophysectomy results in a loss of ability to survive in fresh water by the euryhaline cyprinodont, *Fundulus heteroclitus*. The average survival time is 6–7

days for salt water-adapted fish. Preadaptation in fresh water before operation may prolong survival but does not prevent ultimate death.

2. Hypophysectomized fish are unable to survive in salinities up to 13 ‰.

3. Symptoms of death are those of asthenia accompanied by a slight weight increase, averaging 4.3% in fish not being handled daily.

4. Serum chloride determinations showed that normal fish in either fresh or salt water maintain a uniform level of *ca.* 0.886 gm.% NaCl. Serum of hypophysectomized fish in salt water is not significantly lower (*ca.* 0.817 gm.%). Hypophysectomized fish dying in fresh water have only *ca.* 0.383 gm.%.

5. Replacement therapy with *Fundulus* pituitary brei enabled hypophysectomized fish to survive in fresh water. Pituitary extract from *Perca flavescens,* a strictly fresh water species, was partially effective, whereas extracts from glands of *Pollachius virens,* a marine species, had no beneficial influence.

6. Injections of ACTH, GH, TSH, thyroxin, DOCA, posterior lobe extract, and an ACTH-GH-TSH combination failed to enable the fish to survive in fresh water.

7. The data presented above appear to indicate that the pituitary of *F. heteroclitus* secretes an unknown factor(s) which regulates salt balance in that fish in fresh water. This factor is apparently lacking in the stenohaline marine species, *P. virens,* but may be present in the fresh water species, *P. flavescens.*

8. Hypophysectomy has no effect on the cytology of the chloride cells either in the active (sea water) or regressed (fresh water) condition.

9. Mucous cells were more abundant in the gills of normal fish when adapted to fresh water than when adapted to salt water. Hypophysectomy resulted in atrophy of the mucous cells in either medium and a decreased abundance in fish dying in fresh water. The abundance and condition of the mucous cells was restored to normal in hypophysectomized fish which were induced to survive in fresh water by injections of *Fundulus* pituitary brei.

10. The data suggest that the chloride cells are not concerned in the failure of hypophysectomized fish to survive in fresh water, but that atrophy of the mucous cells may be involved.

LITERATURE CITED

Abramowitz, A. A., 1937. The opercular approach to the pituitary. *Science,* **85**: 609.

Arvy, L., M. Fontaine and M. Gabe, 1954. Action des solutions salines hypertonique sur le système hypothalamo-hypophysaire, chez *Phoxinus laevis,* Agass., et chez *Anguilla anguilla,* L. *C. R. Soc. Biol.,* **148**: 1759–1761.

Arvy, L., M. Fontaine and M. Gabe, 1955. Actions des solutions saline hypertoniques sur le neurosecrétion hypothalamique chez l'anguille hypophysectomisée. *C. R. Soc. Biol.,* **149**: 225–228.

Baker, B. L., and G. D. Abrams, 1955. Growth hormone (somatotropin) and the glands of the digestive system. Internat. Symp. *The Hypophyseal Growth Hormone, Nature and Actions* (R. W. Smith, O. H. Gaebler, C. N. H. Long, Eds.) Blakiston, New York: 107–122.

Baldwin, E., 1937. Introduction to comparative biochemistry. Cambridge University Press, London. Pp. 17–33.

Bargmann, W., 1954. Die endokrine Funktion neurosekretorische Zellen bei Wirbeltieren. *Pubbl. Staz. Zool. Napoli,* **24**, *Suppl.:* 11–14.

Black, V. S., 1951. Some aspects of the physiology of fish. II. Osmotic regulations in teleost fishes. *Univ. Toronto Biol. Ser.,* **59**: 53–89.

Boucher-Firly, S., 1935. Recherches biochimique sur les téléostéens apodes (anguille, congre, murène). *Ann. Instit. Occangr.* n.s., **15**: 108–113.

Boyd, E. M., and M. Dingwall, 1939. The effect of pituitary (posterior lobe) extract on the body water of fish and reptiles. *J. Physiol.*, **95**: 501–507.

Burgess, W. W., A. M. Harvey and E. K. Marshall, Jr., 1933. Site of the antidiuretic action of pituitary extract. *J. Pharmacol.*, **49**: 237–249.

Callamand, O., 1943. L'anguille Européene (*Anguilla anguilla*, L.). Les bases physiologiques de sa migration. *Ann. Instit. Occangr. Monaco*, **21**: 361–440.

Callamand, O., M. Fontaine, M. Olivereau and A. Raffy, 1951. Hypophyse et osmorégulation chez les poissons. *Bull. Instit. Occangr. Monaco No. 984:* 1–7.

Chavin, W., 1954. The role of the pituitary-adrenal mechanism in the reappearance of melanin and melanophores in the goldfish, *Carassius auratus*, L. Dissertation, New York University.

Copeland, D. E., 1948. The cytological basis of chloride transfer in the gills of *Fundulus heteroclitus*. *J. Morph.* **82**: 201–227.

Copeland, D. E., 1950. Adaptive behavior of the chloride cell in the gill of *Fundulus heteroclitus*. *J. Morph.*, **87**: 369–380.

Coventry, F. H., V. E. Shelford and L. F. Miller, 1935. The conditioning of a chloramine treated water supply for biological purposes. *Ecology*, **16**: 60–66.

D'Angelo, S. A., 1955. Pituitary regulation of thyroid gland function. Brookhaven Symp., *The Thyroid:* 9–27.

Drilhon, A., 1942. Destruction de la moelle et adaptation aux changements de salinité d'un poisson homeoosmotique (carpe). *C. R. Acad. Sci. Paris*, **214**: 575–577.

Firly, S., 1932. Influence des variations de salinité sur la pression osmotique des civelles. *C. R. Soc. Biol.*, **110**: 247–248.

Fontaine, M., 1953. Equilibre hydrominerale et quelques particularités de sa régulation chez les vertebres. *Arch. Sci. Physiol.*, **7**: C55–77.

Fontaine, M., 1956. The hormonal control of water and salt-electrolyte metabolism in fish. *Mem. Soc. Endocrin.*, **5**. (In press).

Fontaine, M., and S. Boucher-Firly, 1934. Recherches sur la reserve alcaline du sang de poissons, ses variations au cours des changements de salinité. *Bull. Instit. Oceanogr. Monaco No. 646:* 1–12.

Fontaine, M., and O. Callamand, 1948. Nouvelles recherches sur le determinisme physiologique de l'avalaison des poissons migrateurs amphibiotiques. *Bull. Mus. Nation. Hist. Nat. Paris* (2), **20**: 317–320.

Fontaine, M., O. Callamand and M. Olivereau, 1949. Hypophyse et euryhalinité chez l'anguille. *C. R. Acad. Sci. Paris*, **228**: 513:514.

Fontaine, M., and A. Raffy, 1950. Le facteur hypophysaire de rétention d'eau chez les téléostéens. *C. R. Soc. Biol. Paris*, **144**: 6–7.

Garrey, W. E., 1905. The osmotic pressure of sea water and the blood of marine animals. *Biol. Bull.*, **8**: 257–270.

Gaunt, R., J. H. Birnie, W. R. Ross, W. J. Eversole and C. M. Osborn, 1951. The adrenal cortex in water metabolism. AAAS Symp. *Pituitary Adrenal Function.* (R. C. Christman, Ed.) AAAS, Washington: 102–108.

Getman, H. C., 1950. Adaptive changes in the chloride cells of *Anguilla rostrata*. *Biol. Bull.*, **99**: 439–445.

Glick, D., 1949. Techniques of histo- and cytochemistry. Interscience, New York: 166 et seq.

Gorbman, A., and O. Berg, 1955. Thyroid function in the fishes *Fundulus heteroclitus*, *F. majalis*, and *F. diaphanus*. *Endocrin.*, **56**: 86–92.

Grafflin, A. L., 1931. Urine flow and diuresis in marine teleosts. *Amer. J. Physiol.*, **97**: 602–610.

Grafflin, A., 1935. Chloride and total osmotic pressure in the blood of marine teleosts. *Biol. Bull.*, **69**: 245–258.

Grafflin, A. L., 1937. The problem of adaptation to fresh and salt water in the teleosts viewed from the standpoint of the structure of the renal tubules. *J. Cell. Comp. Physiol.*, **9**: 469–476.

Hall, B. A., I. E. Gray and S. Lepkovsky, 1926. The influence of asphyxiation on the blood constituents of marine fishes. *J. Biol. Chem.*, **67**: 549–554.

Hawk, P. B., B. L. Oser and W. H. Summerson, 1947. Practical physiological chemistry. Blakiston, Philadelphia: 574–575.

Henschel, 1934–35. Wasseraushalt und Osmoregulation von Scholle und Flunder. *Wissenschaftliche Meeresuntersuchunges*, **22**: 91–121.

Hoar, W. S., 1951. Some aspects of the physiology of fish. I. Hormones in fish. *Univ. Toronto Biol. Ser.*, **59**: 1–51.

Kalashnikov, G. N., 1939. "The composition of the blood of fish" (In Russian). *Uchenye Zapiski MGU Hydrobiol.*, **33**: 65–103.

Kalashnikov, G. N., and S. N. Skadovskii, 1948. "Ecological and physiological study of the sevriuga during the period of reproduction under natural and experimental conditions" (In Russian). *Zool. Zhurnal.*, **27**: 513–524.

Keys, A., and J. B. Bateman, 1932. Branchial responses to adrenalin and to pitressin in the eel. *Biol. Bull.*, **63**: 327–336.

Keys, A., and E. N. Wilmer, 1932. "Chloride secreting cells" in the gills of fishes, with special reference to the common eel. *J. Physiol.*, **76**: 368–378.

Kirshenblatt, J. D., 1952. "The action of steroid hormones on female vy'un" (In Russian). *Doklady Akad. Nauk*, **83**: 629–632.

Koch, H. J., and M. J. Heuts, 1942. Influence de l'hormone thyroidienne sur la régulation osmotique chez *Gasterosteus aculeatus*, L., form *gymnurus*, Cuv. *Ann. Soc. R. Zool. Belg.*, **73**: 165.

Krogh, A., 1939. Osmotic regulation in aquatic animals. Cambridge University Press, London.

Levinsky, N. G., and W. H. Sawyer, 1952. Influence of the adenohypophysis on the frog water balance response. *Endocrin.*, **51**: 110–116.

Liu, C. K., 1942. Osmotic regulation and "chloride secreting cells" in the paradise fish—*Macropodus opercularis*. *Sinensia*, **13**: 15–20.

Matthews, S. A., 1933. Color changes in *Fundulus* after hypophysectomy. *Biol. Bull.*, **64**: 315–320.

Martini, L., and C. Morpurgo, 1955. Neurohumoral control of the release of adrenocorticotropic hormones. *Nature*, **175**: 1127–1128.

Nishida, H., 1953. The cytohistochemical observations on the gland cells of the branchial epidermis with the comparison of two types of Oncorynchus masou, landlocked and sea run forms. (In Japanese with English summary). *Sci. Rpts. Hokkaido Fish Hatchery*, **8**: 33-38.

Pickford, G. E., 1953. The hypophysectomized male killifish. *Bull. Bingham Oceanogr. Coll.*, **14** (2): 5-41.

Pickford, G. E., 1954a. The response of hypophysectomized male killifish to purified fish growth hormone, as compared with the response to purified beef growth hormone. *Endocrin.*, **55**: 274-287.

Pickford, G. E., 1954b. The response of hypophysectomized male killifish to prolonged treatment with small doses of thyrotropin. *Endocrin.*, **55**: 589–592.

Puschel, J., 1928. Blutuntersuchungen bei einem Süsswasserteleostier (*Tinca vulgaris*, Cuv.). *Zeitschr. f. vergl. Physiol.*, **7**: 606–610.

Sawyer, W. H., 1956. The hormonal control of water and salt-electrolyte metabolism with special reference to the amphibia. *Mem. Soc. Endocrin.*, **5** (In press).

Smith, D. C. W., 1956. The role of the endocrine organs in the salinity tolerance of trout. *Mem. Soc. Endocrin.*, **5** (In press).

Stevens, C. E., and C. P. Leblond, 1953. Renewal of the mucous cells in the gastric mucosa of the rat. *Anat. Rec.*, **115**: 231–243.

Sumner, F. B., 1906. The physiological effects upon fishes of changes in density and salinity of water. *Bull. U. S. Bur. Fish.*, **25**: 53–108.

Vars, H. M., 1934. Blood studies on fish and turtles. *J. Biol. Chem.*, **105**: 135–137.

White, H. L., 1955. Growth hormone and renal function. Internat. Symp., *The Hypophyseal Growth Hormone, Nature and Actions* (R. W. Smith, O. H. Gaebler, C. N. H. Long, Eds.). Blakiston, New York, 178–186.

Wilhelmi, A. E., G. E. Pickford and W. H. Sawyer, 1955. Initiation of the spawning reflex response in *Fundulus* by the administration of fish and mammalian neurohypophyseal preparations and synthetic oxytocin. *Endocrin.*, **57**: 243–252.